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PRINCIPAL INVESTIGATOR: Shin-ichi Takayama, M.D., Ph.D.

CONTRACTING ORGANIZATION: The Burnham Institute
La Jolla, California 92037

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Shin-ichi Takayama, M.D., Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
The Burnham Institute
La Jolla, California 92037

E-Mail: stakayama@burnham.org

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**13. Abstract** ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
BAG-family proteins regulate diverse cellular functions, including cell survival, cell proliferation, and cell motility. BAG-family proteins contain a conserved domain that allows them to bind 70-kD heat shock (Hsp70) family molecular chaperones and regulate their activity. Structural analysis of the Hsc70-binding BAG domain of BAG1 has revealed an anti-parallel two helix bundle, proceeded by an additional long α-helix. Site-directed mutagenesis has confirmed that the polar surfaces of the α-helices in the BAG domain are directly involved in chaperone binding, which has been confirmed by NMR experiments. Similarly, an ~80 amino acid region (229-308) of Hsc70 has been determined to represent a minimal domain sufficient for binding the BAG domain. In addition to the Hsp70-binding domain, BAG-family proteins also contain a diversity of additional domains, which allow them to interact with specific target proteins or which target them to specific locations within cells. The BAG-family proteins operate as bridging molecules that recruit molecular chaperones to target proteins and ultimately affecting diverse cellular behaviors including cell division, migration, differentiation, and death in cancer cells. Recently BAG3 was reported as a regulator of cell growth. Our preliminary result also shows BAG3 might have a tumorigenic activity.

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# BODY

## OBJECTIVES

The original funded objectives of the project were to:

1) Deduce the complete primary amino-acid sequences of BAG3, BAG4, and BAG5.
2) Determine the biochemical effects of BAG-family proteins on Hsc70 chaperone function.
   a) Subclone cDNAs into pGEX4T-1 for production of FST fusion proteins
   b) Produce and purify BAG-family proteins from bacteria
   c) Perform biochemical assays of BAG-family protein effects on Hsc70 chaperone activity (refolding assays), ATPase activity, and ADP-ATP exchange
3) Map the domains within BAG-family proteins which are needed for interaction with Hsc70.
   a) Create deletion mutant of BAG1 family proteins by PCR mutagenesis of cDNAs
   b) Subclone into pGEX and yeast two hybrid plasmids
   c) Perform Hsc70 interaction assays
4) Examine effects of BAG-family proteins on Estrogen Receptor Function.
5) Explore the effects of BAG-family proteins on in vitro behaviors of breast cancer cells.
6) Determine the incidence of BAG-family protein expression in breast cancers.

## PROGRESS

**Objective #1.** Deduce the complete primary amino-acid sequences of BAG3, BAG4, and BAG5.

This aim has been completed and reported in the first annual report.

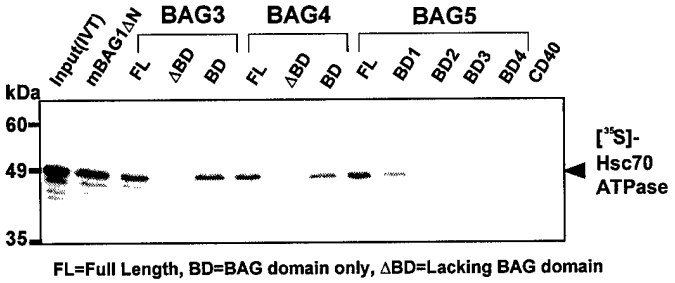
**Objective #2.** Determine the biochemical effects of BAG-family proteins on Hsc70 chaperone function.

All GST fusion proteins show degradation problems. Objective 3 should be completed first and then we will attempt Objective 2.

**Objective #3.** Map the domains within BAG-family proteins which are needed for interactions with Hsc70.

Full-length BAG3 and the fragment containing only the BAG domain (BD) bound to GST-Hsc70, whereas BAG3 lacking the BAG domain (ΔBD) did not. Similar results were obtained with BAG4. The BAG5 protein contains four potential BAG domains. Expression of each of these individually demonstrated that only

the first (most N-terminal) of the BAG domains binds Hsc70 in vitro. Similar results were obtained by yeast two-hybrid assays (not shown).




**BAG domain binds ATPase domain of Hsc70 in vitro.** GST fusion proteins were immobilized on glutathione-Sepharose beads as indicated (5 µg) and mixed in a volume of 0.1 ml with 1 µl of in vitro translated $^{35}$S-L-methionine labeled ATPase domain of Hsc70. After 1hr incubation at 4°C, beads was washed 3 times with 1 ml of ice-cold 0.5% NP40, 20 mM HEPES (pH 7.7), 142 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA. Following SDS-PAGE separation, autoradiography was used to visualize the proteins. The GST fusion proteins represented full-length BAG3, 4, or 5, or fragments corresponding only to the BAG Domains (BD), or fragments lacking the BAG-domains (ΔBD). Note that BAG5 contains four potential BAG-domains, labeled BD1, BD2, BD3, and BD4. GST-CD40 served as a negative control

**Structure and mutational analysis of BAG domain.** Recently, in collaboration with Dr. Ely at our institution, we completed a NMR solution structure of a fragment of the mouse BAG1 protein (residues 90-219), representing the last 130 amino acids, which encompasses the BAG-domain and upstream region proximal to the UBL domain. The structure reveals three long α-helices (α1, α2, and α3), with the last two α-helices (α2 and α3) corresponding to the BAG domain. Alignment of the sequences corresponding to the BAG domains of the other BAG-family members, and secondary structure prediction algorithms, suggest that most of the BAG domains share this two α-helix structure. Thus, the structural information derived for BAG1 should be relevant to our efforts to understand BAG family proteins through mutational analysis. Paper is attached with this report (Briknarova et al. 2001).

| Mutant | α-helix | Residues | Hsp70 binding | AR Co-activation |
|---|---|---|---|---|
| Wild Type | NA | NA | ++ | ++ |
| H1A | α1 | E115A, K116A, N119A | ++ | NA |
| H1B | α1 | E123A, K126A | ++ | NA |
| H2A | α2 | D149A, R150A | ++ | NA |
| H2B | α2 | E157A, K161A | - | - |
| H3A | α3 | Q190A | ± | NA |
| H3B | α3 | D197A, Q201A | - | - |

**Table 1. Characterization of BAG domain mutants.** The mouse BAG1 protein (residues 90-219) or various mutants as indicated in the Table were expressed as GST fusion protein in bacterial and affinity purified. GST-BAG1 fusion proteins were assayed for ability to bind Hsc70/ATPase in vitro. To correlate in vitro binding with in vivo activity, some of the same mutants were produced in the BAG domain of the human BAG1L protein and their function was assessed by transient transfection reporter gene assays, using co-activation of the androgen receptor (AR) as a convenient read-out. Results are compared relative to wild-type BAG1. NA = not applicable/not tested.

**Objective #4.** Examine effects of BAG-family proteins on Estrogen Receptor Function.

For the model system of this analysis, we examined BAG1 on ER reporter gene assay. Still working on BAG1 and ER.

**Objective #5.** Explore the effects of BAG-family proteins on in vitro behaviors of breast cancer cells.

Our initial investigation about BAG2-3 represent over-expression of BAG3 protein in breast cancer cell lines (described in the last annual report).

**BAG3 displays transforming activity in 3T3 cell transfection assays.** The effects of BAG3 and various other proteins on morphological transformation of Balb/c 3T3 cells were tested by transfection assay. pcDNA3 parent (Control) plasmid and a plasmid encoding the BAG1L protein failed to induce significant numbers of transformed foci, whereas oncogenic Ras (Val12) served as a positive control for the assay. Both full-length BAG3 and a mutant lacking the C-terminal Hsc70-binding domain induced a significant increase in transformed foci in this assay. These data imply that BAG3 has at least modest transforming activity and that Hsc70-binding is not required for this activity.

Over-expression of BAG3 protein are observed in several cancer cell lines, including breast cancer cell lines (see result in the task6). The human BAG3 protein is a cytosolic protein of 575 amino-acids length, which contains a WW domain, followed by a proline rich region, and then the Hsc70/Hsp70-binding BAG domain. WW domains are found in several proteins of relevance to signal transduction, and are known to bind XPPXY motifs. The proline-rich region of BAG3 contains several PXXP motifs, which are known to interact with SH3 domains, and indeed BAG3 has been reported to bind the SH3 protein, Phospholipase Cγ (PLCγ). These structural features of the BAG3 protein suggest that it is involved in some aspect of signal transduction. In this regard, using two-hybrid methods, we have identified candidate BAG3 binding proteins, including a Guanine Nucleotide Exchange Factor, which is believed to regulate small GTPases, and we have confirmed its association with BAG3 in vitro and in vivo. Our hypothesis therefore is that BAG3 coordinates signals related to cell adhesion, cytoskeleton regulation, or related processes. Therefore we expect BAG3 may be an important regulator of malignant transformation, tumor invasion or metastasis. Based on these preliminary results, we would like to examine BAG3 first (BAG2, BAG4, BAG5 will be analyzed later) in breast cancer biology.

**Objective #6.** Determine the incidence of BAG-family protein expression in breast cancers.

BAG2, BAG3, BAG4 and BAG5 anti-sera have been generated in my lab. Specificity of these antibodies has been checked by western blot analysis. We recently found that Cytosolic immunostaining for BAG1 was up-regulated in 79 (65%) of 122 invasive breast cancers (P <.001) compared with normal breast. Elevated BAG1 was significantly associated with longer DMFS and OS, overall (stages 1 and II) and in node-negative (stage I only) patients, on the basis of univariate and multivariate analyses (DMFS, P =.005; OS, P =.01, in multivariate analysis of all patients; DMFS, P =.005; OS, P =.001, in multivariate analysis of node-negative patients). All other biomarkers failed to reach statistical significance in multivariate analysis. Clinical stage was an independent predictor of OS (P =.04) and DMFS (P =.02). These findings provide preliminary evidence that BAG1 represents a potential marker of improved survival in early-stage breast cancer patients, independent of the status of axillary lymph nodes. See attached paper, Turner et al. J Clin Oncol, 2001.

**KEY RESEARCH ACCOMPLISHMENTS**

1) We determined structure of BAG domains in BAG1 by NMR analysis.
2) GST fusion protein of BAG domain from BAG3 through BAG5 was purified and we found that not all of these BAG domains can bind to ATPase domain of Hsp70.
3) Cytosolic immunostaining for BAG1 was up-regulated in invasive breast cancer comparing with normal breast.
4) Rabbit antisera have been generated and characterised against all protein of BAG family.
5) BAG3 represent over expression in breast cancer cell lines and shows tumorigenic activity in Balb/c 3T3 cell line.
6) We cloned guanine nucleotide exchange factor as a BAG3 binding protein.

**PUBLICATIONS**

1) Brikanova et al Nature Structure Biology, 2001
2) Turner et al, Journal of Clinical Oncology, 2001
3) Takayama et al, Nature Cell Biology, Review, submitted

## CONCLUSION

Structural analysis of the Hsc70-binding BAG domain of BAG1 has revealed an anti-parallel two helix bundle, proceeded by an additional long α–helix. Site-directed mutagenesis has confirmed that the polar surfaces of the α–helices in the BAG domain are directly involved in chaperone binding, which has been confirmed by NMR experiments. Similarly, an ~80 amino acid region (229-308) of Hsc70 has been determined to represent a minimal domain sufficient for binding the BAG domain. The conserved BAG domains of BAG1 family protein from plant and yeast also bind the Hsc70 ATPase domain.

In addition to the Hsp70-binding domain, BAG-family proteins also contain a diversity of additional domains, which allow them to interact with specific target proteins or which target them to specific locations within cells. The BAG-family proteins operate as bridging molecules that recruit molecular chaperones to target proteins, presumably modulating protein functions through alterations in their conformations, and ultimately affecting diverse cellular behaviors including cell division, migration, differentiation, and death. Emerging knowledge about BAG-family proteins suggests a mechanism for influencing signal transduction through non-covalent post-translational modifications.

Cytosolic immunostaining for BAG1 was up-regulated in 79 (65%) of 122 invasive breast cancers ($P < .001$) compared with normal breast. These findings provide preliminary evidence that BAG1 represents a potential marker of improved survival in early-stage breast cancer patients, independent of the status of axillary lymph nodes.

The human BAG3 protein is a cytosolic protein of 575 amino-acids length which contains a WW domain, followed by a proline rich region, and then the Hsc70/Hsp70-binding BAG domain. WW domains are found in several proteins of relevance to signal transduction, and are known to bind XPPXY motifs. The proline-rich region of BAG3 contains several PXXP motifs, which are known to interact with SH3 domains, and indeed BAG3 has been reported to bind the SH3 protein, Phospholipase Cγ (PLCγ). These structural features of the BAG3 protein suggest that BAG3 is involved in some aspect of signal transduction. In this regard, using two-hybrid methods, we have identified candidate BAG3 binding proteins, including a Guanine Nucleotide Exchange Factor and we have confirmed its association with BAG3 in vitro and in vivo. Our hypothesis therefore is that BAG3 co-ordinates signals related to cell adhesion, cytoskeleton regulation, or related processes. Consistent with this hypothesis, over-expression of BAG3 is transforming in 3T3 fibroblasts. Interestingly, our preliminary data also suggest that BAG3 is over-expressing in cancer cells. As such, BAG3 may be an important regulator of malignant transformation, tumor invasion or metastasis.